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1. Your reference — P.77069 GCW

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2. Patent application number *(The Patent Office will fill in this part)* — 31 AUG 1999

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United Kingdom

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — SCREEN FOR AXON VIABILITY

5. Name of your agent *(if you have one)* — J A KEMP & CO

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Number of earlier application	Date of filing *(day / month / year)*

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   b) there is an inventor who is not named as an applicant, or
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   See note (d))

YES

SCREEN FOR AXON VIABILITY

Technical field of the invention

This invention relates to methods for assaying for axon viability and to methods for screening for substances which protect axons from loss of viability.

Background to the invention

Axons in CNS white matter become damaged in various debilitating conditions affecting humans, including stroke, trauma and multiple sclerosis (Stys, 1998; Trápp et al., 1998). The underlying mechanisms, however, have not been investigated as extensively as those causing damage to grey matter. In part at least, this is attributable to the technical difficulties of studying white matter pathology. The available information on white matter axons has so far come mainly from electrophysiological experiments on the rat isolated optic nerve preparations, in which the degree of recovery of the compound action potential following transient anoxia is used as an index of viability (Stys, 1998). A similar method has been applied to traumatic damage in the spinal cord (Agrawal & Fehlings, 1996).

A quantitative morphometric approach for analysing white matter axon pathology has recently been developed and used to study the mechanisms of rat optic nerve axon degeneration resulting from transient oxygen- and glucose-deprivation (OGD) *in vitro* (Garthwaite *et al.*, 1999). The results suggest a mechanism similar to that proposed to explain anoxic axonal damage (Stys, 1998), namely that excessive influx of Na^+ through voltage-dependent Na^+ channels is followed by lethal Ca^{2+} overload of the axoplasm through reversal of the Na^+-Ca^{2+}-exchanger located in the cell membrane. The histological method, however, suffers from the disadvantage of not recording axonal function and so interpretations based purely on morphological criteria may be misleading.

Nitric oxide (NO) functions as a diffusible second messenger molecule in most areas of the central nervous system (CNS). It is generated from L-arginine by NO synthase enzymes, the neuronal isoform of which is functionally and physically associated with the N-methyl-D-aspartate type of glutamate receptor in many brain

of viability comprising:

(i) contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability;

(ii) determining the viability of the axon by a method according to any one of the preceding claims; and

(iii) determining thereby whether the test substance can protect the axon from loss of viability;

- a substance identified by a method for identifying a substance capable of protecting an axon from loss of viability;
- a substance of the invention for use in a method of treatment of the human or animal body by therapy;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease;
- a method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance of the invention; and
- a method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance of the invention.

Brief description of the figures

Figure 1 shows (a) DEA/NO concentration-response curve for cGMP

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

This assay for axon viability is significant, as no other simple methods for assessing white matter axon viability are presently available.

In principle the assay for determining the viability of an axon may be carried out to determine the viability of any axon. However, the assay is particularly suitable for determining the viability of white matter axons. White matter is an area of the nervous system, containing abundant myelinated axons and is therefore light in colour. The central nervous system comprising the brain and spinal cord and the peripheral nervous system both contain white matter and axons from these sources may be used in the assay of the invention. Axons from the optic nerve are particularly suitable.

In principle the assay may be carried out using a single axon. However, in practice it is more convenient to use more than one axon in a single assay. Typically, a population of axons, for example a nerve, is used. The viability determined when more than one axon is used will represent an average viability for the population of axons used.

In viable axons, NO activates sGC, leading to an increase in cGMP formation, which in turn leads to the modulation of the activity of a number of cGMP targets. A viable axon may thus be identified by determining whether this pathway is functional in that axon. The activity of sGC before and after contacting an axon with a substance capable of stimulating sGC may be determined in order to determine whether sGC activity is stimulated, thereby to determine whether the axon is viable.

Any suitable format may be used for carrying out the assay of the invention. Generally, the assay is carried out *ex vivo* and under physiologically acceptable conditions; that is, under conditions that would be expected to support axon survival. It will often be convenient to carry out the assay in an aqueous medium, for example a physiologically acceptable buffer.

Typically, the assay is initiated by contacting an axon with a substance that is capable of stimulating sGC. Such a substance is generally one which under normal physiological conditions is capable of activating sGC in a viable axon. Suitable

enzyme-linked immunoassays (ELISA) and immunohistochemistry may be used. If radioimmunoassays or ELISA are used, typically the total protein content of the tissue is also assayed. In that way the amount of cGMP in a sample can be expressed per amount of protein. Radioimmunoassays, ELISA and immunohistochemistry may all be carried out using anti-cGMP antibodies. Any suitable antibodies may be used. For example, suitable antibodies for use in immunohistochemistry are described in De Vente *et al.* (1987). The above techniques are all well known to those skilled in the art.

cGMP is broken down in cells by the action of phosphodiesterases (PDEs). Therefore, the rate of cGMP accumulation is the difference between its rate of formation by sGC and its rate of destruction by PDEs and if PDE activity is high, cGMP accumulation may not be observed. Thus, PDE inhibitors, for example non-selective PDE inhibitors such as 3- isobutyl-1-methylxanthine (IBMX), may also be added to the assay. In the presence of such inhibitors the rate of cGMP accumulation is equal to the rate of cGMP formation.

The activity of sGC may also be determined indirectly by measuring, for example, the activity of a target of cGMP. Thus, for a viable axon sGC stimulation may be determined by measuring any modulation in the activity of a cGMP target. A number of cGMP targets are known. For example, cGMP activates cGMP dependent protein kinase as well as ion channels. Additionally, the activities of phosphodiesterases are modulated in response to cGMP. Measurement of any of these targets may be used to, indirectly, determine whether sGC is stimulated.

Appropriate control experiments may be carried out when performing the assay of the invention. For example, the assay will be carried out in both the absence and presence of a substance capable of stimulating sGC. Additionally, if cGMP increase or modulation of a cGMP target are measured, the involvement of sGC stimulation may be confirmed by carrying out the assay in the presence of an inhibitor of sGC, for example 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. If sGC is involved in the elevation of cGMP levels in response to NO stimulation, the presence of an sGC inhibitor will reduce the cGMP response observed in the absence of that inhibitor.

protecting an axon from loss of viability, a "protectant". Thus, substances may be identified which preserve axon viability under conditions that would typically lead to axon damage or axon death. Substances identified by such methods may be useful in the prevention and/or treatment of conditions in which damage to or death of axons, in particular CNS white matter axons, is implicated.

Any suitable format may be used for identifying a substance capable of protecting an axon from loss of viability. The assay is, however, typically carried out in an aqueous medium and preferably in a single well of a plastics microtitre plate, so that high through-put screening for protectants may be carried out.

Typically an axon is contacted with a test substance under conditions that, in the absence of the test substance, would lead to a reduction in viability of that axon. Suitable conditions are described above. The viability of an axon may be determined using the viability assay of the invention and this will allow the ability of a test substance to prevent loss of viability to be ascertained.

Suitable control experiments may be carried out. For example, the method may be carried out in the absence of a test substance in order to determine any basal level of sGC stimulation for non-viable axons. Positive control assays may be carried out using the known neuroprotectants, lamotrigine, BW619C89 and BW1003C78 (Xie *et al.*, 1995; Xie and Garthwaite, 1996; Meldrum *et al.*, 1992)

Combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries may be screened for activity as protectants in assays such as those described above. The candidate substances may be chemical compounds. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition tested individually. Suitable candidate substances include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies).

A substance which is capable of protecting an axon from a loss of viability, a "protectant", is one which causes a measurable increase in axon viability in the method described above. Preferred substances are those cause an increase in axon viability of at least 10%, at least 25%, at least 50%, at least 100% at least 200%, at

aqueous or oily suspensions, dispersible powders or granules. The protectants may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The protectants may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a protectant for use in the treatment of a condition associated with white matter damage will depend upon factors such as the nature of the exact protectant, whether a pharmaceutical or veterinary use is intended, etc. A protectant may be formulated for simultaneous, separate or sequential use.

A protectant is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film-coating processes.

Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired,

After 1-2 h preincubation in aCSF, test nerves were transferred into aCSF lacking glucose and gassed with 5% CO_2 in N_2 for 1 h, a period shown previously to result in irreversible damage to the majority of axons (Garthwaite *et al.*, 1999). Afterwards, the nerves were given a 90 min recovery period in normal aCSF. Modified aCSF and putative axonoprotective compounds were present from 15 min before until 15 min after OGD.

cGMP accumulation

Nerves, with or without a preceding 1 h exposure to OGD (plus 90 min recovery) were exposed to the nitric oxide (NO) donor, DEA/NO (2,2-diethyl-1-nitroso-oxyhydrazine) for 5 min. They were then inactivated in boiling hypotonic buffer and their protein and cGMP contents measured using the automated Lowry method and radioimmunoassay, respectively, as described (Garthwaite & Garthwaite, 1987). The general phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM) was added 10 min before the exposure to the NO donor, except where indicated. Results are given as means ± SEM and were evaluated using the unpaired Student's *t*-test (2-tailed), $P < 0.05$ being considered significant.

Histology and cGMP Immunohistochemistry

Conventional histology was carried out on semithin sections of resin-embedded nerves as described previously (Garthwaite *et al.*, 1999). For cGMP immunohistochemistry, nerves, with or without various treatments (as described in the text) were fixed in ice-cold, freshly-depolymerised paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) for 2 h, processed as described before (Southam & Garthwaite, 1993), and then frozen on a cryostat chuck and sectioned at 10 µm intervals. Some nerves were embedded in resin (Durcupan) using conventional methods and cut into 1 µm thick sections. cGMP immunostaining was conducted using a sheep anti-cGMP antibody (Tanaka *et al.*, 1997). Briefly, the sections were incubated with primary antibody (1:80,000) overnight at 4°C . They were then incubated at room temperature with rabbit biotinylated anti-sheep antibody (1:1000; 1 h) followed by Vector stain ABC elite kit (30 min) and then 3.3'-diaminobenzidine

pmol/mg protein, was only 32 ± 3 pmol/mg protein (n = 4), implying a high endogenous phosphodiesterase activity.

Conventional histology of resin-embedded nerves showed that, under control conditions, axons and glial cells (astrocytes and oligodendrocytes) were well preserved in incubated optic nerves (Fig. 2a,d), in agreement with previous findings (Waxman *et al.*, 1992; Garthwaite *et al.*, 1999). To locate the sites of cGMP accumulation, immunohistochemistry was used. In frozen sections from unstimulated nerves (incubated with IBMX), no immunostaining was observed (Fig. 2b). In contrast, exposure to 100 μM DEA/NO (in the presence of IBMX) for 5 min produced powerful staining that was apparently restricted to axons (Fig. 2c). Higher resolution immunohistochemical staining, carried out on semithin sections from resin-embedded nerves, confirmed the staining to be in axons, with no detectable labelling of myelin or glial cells (Fig. 2e,f).

When optic nerves were subjected to 1 h of OGD followed by 90 min recovery in normal aCSF, histology showed abundant axonal swelling (Fig. 2g). The biochemically-measured cGMP response to DEA/NO (100 μM) in nerves previously subjected to OGD was reduced by about 80% (Fig. 1b & 3) and cGMP immunohistochemistry of such nerves showed a marked loss of labelled axons; although there remained a few that stained normally (Fig. 2j).

To further examine the validity of the cGMP response as a marker of axon viability, manoeuvres found previously to reduce or eliminate anoxia-induced loss of the optic nerve compound action potential (Stys, 1998) or OGD-induced axon pathology (Garthwaite *et al.*, 1999) were tested. Complete preservation of the cGMP response was achieved if OGD was imposed in Ca^{2+}-free aCSF or in the presence of the voltage-dependent Na^+ channel inhibitor, tetrodotoxin (TTX, 1 μM); Na^+-free aCSF was less effective, affording only 60% protection (Fig. 1b). Control experiments showed that the cGMP response of nerves exposed to Ca^{2+}-free or Na^+-free aCSF, or TTX, for the same intervals (but without OGD) were normal (n = 4, results not shown). When examined under the microscope, TTX prevented OGD-induced axonopathy (Fig. 2i) and, in parallel, OGD-induced loss of cGMP immunostaining of the axons following exposure to DEA/NO (Fig. 2l). Similar

led to cGMP formation specifically in optic nerve axons is surprising for two reasons. First, previous evidence had indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, particularly those mediating glutamatergic neurotransmission (Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997), yet synapses are absent in the optic nerve. Second, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way because, in bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds (Gotzes *et al.*, 1998). This may indicate that NO-sensitive guanylyl cyclase is preferentially targetted to the axons rather than to the somatodendritic regions of these particular neurones. Judging by the large enhancement of NO-induced cGMP accumulation brought about by IBMX, the axons are also likely to be rich in phosphodiesterase activity, supporting the possibility that the expression of the guanylyl cyclase there has functional relevance.

Concerning possible sources of endogenous NO in the optic nerve, there is histochemical evidence that guinea-pig optic nerve astrocytes contain an NO synthase enzyme (Qi & Guy, 1996) but we have been unable to detect the endothelial, neuronal or the inducible NO synthase isoforms in glia or axons of the normal rat optic nerve by immunohistochemistry. Staining for the endothelial isoform in endothelial cells themselves, however, was clearly observed (unpublished observations). Thus, NO derived from endothelial cells might constitute the normal effector for the stimulation of cGMP accumulation in optic nerve axons. If so, this would constitute an unusual pathway for intercellular signalling by NO. Additional sources of NO may be present in pathological conditions since, in human glaucomatous patients, the three different NO synthase isoforms are apparently expressed in optic nerve glia (Neufeld *et al.*, 1997), raising the possibility that this pathway is relevant to disorders of optic nerve function in humans. Understanding the functional consequences of cGMP formation in the axons awaits investigation but, in pilot experiments, we have observed that NO-donors elicit a depolarising response from the optic nerve, suggesting a possible action on axonal ion channels (unpublished observation).

explained by this manoeuvre itself causing influx of Ca^{2+} which could sum with Ca^{2+} coming in via routes other than the Na^+-Ca^{2+}-exchanger during OGD (Stys & Lopachin, 1998).

Two of the pharmacological agents tested, lamotrigine and BW619C89, have been shown by detailed electrophysiological analysis to be use- and voltage-dependent blockers of voltage-dependent Na^+ channels in central neurones and in cell lines expressing type II Na^+ channels (Xie *et al.*, 1995; Xie & Garthwaite, 1996). The third compound, BW1003C87, is likely to have a similar action since it has a closely related structure and it inhibits glutamate release from brain tissue exposed to the Na^+-channel opener, veratrine, but not the release induced by raised K^+ (Meldrum *et al.*, 1992). All three compounds protect grey matter from ischaemia *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). In the present study, BW619C89 protected the axons with a potency and efficacy very similar to those registered by morphometric assay (Garthwaite *et al.*, 1999); the degree of protection achieved by the other compounds, at concentrations shown to be maximally effective, also matched those reported by morphometric assay (Garthwaite *et al.*, 1999). The explanation for the differential protective efficacies of the three structurally-similar molecules towards optic nerve axons (BW619C89>BW1003C87>lamotrigine) awaits investigation but it may relate to a differential blockade of the non-inactivating axonal Na^+ channels that appear responsible for much of the Na^+ influx, at least under conditions of anoxia (Stys *et al.*, 1993). Molecules like BW619C89 which appear able to afford a high degree of protection towards both white matter axons and grey matter subjected to ischaemia-like insults, should, in principle, offer superior treatment for conditions such as stroke than strategies (e.g. glutamate receptor blockade) only capable of protecting grey matter.

In conclusion, in the rat optic nerve, the axons selectively and richly express functional NO receptor protein, enabling them to generate large amounts of cGMP in response to NO. While the functional implications of this response remain to be defined, its existence provides a novel, simple and reliable method for quantitatively assessing axonal viability that is likely prove valuable in studies of the pathogenesis of axonal damage and for assessing axonoprotective measures.

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CLAIMS

1. A method for determining the viability of an axon comprising:
   (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);
   (ii) determining whether sGC is stimulated in the axon; and
   (iii) determining thereby whether the axon is viable.

2. A method according to claim 1, wherein the axon is a white matter axon.

3. A method according to claim 2, wherein the white matter axon is from the optic nerve, the brain or the spinal cord.

4. A method according to any one of the preceding claims, wherein step (i) is carried out by contacting the axon with nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO.

5. A method according to claim 4 wherein NO is provided in the form of an NO donor.

6. A method according to claim 5, wherein the NO donor is 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO).

7. A method according to any one of the previous claims, wherein step (ii) is carried out by determining whether cGMP generation by the axon increases.

8. A method according to claim 8, wherein the generation of cGMP is determined by radioimmunoassay or immunocytochemistry.

9. A method according to claim 7 or 8, wherein a viable axon is one which

for use in the treatment of a condition associated with white matter damage.

17. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

18. A method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

19. A method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

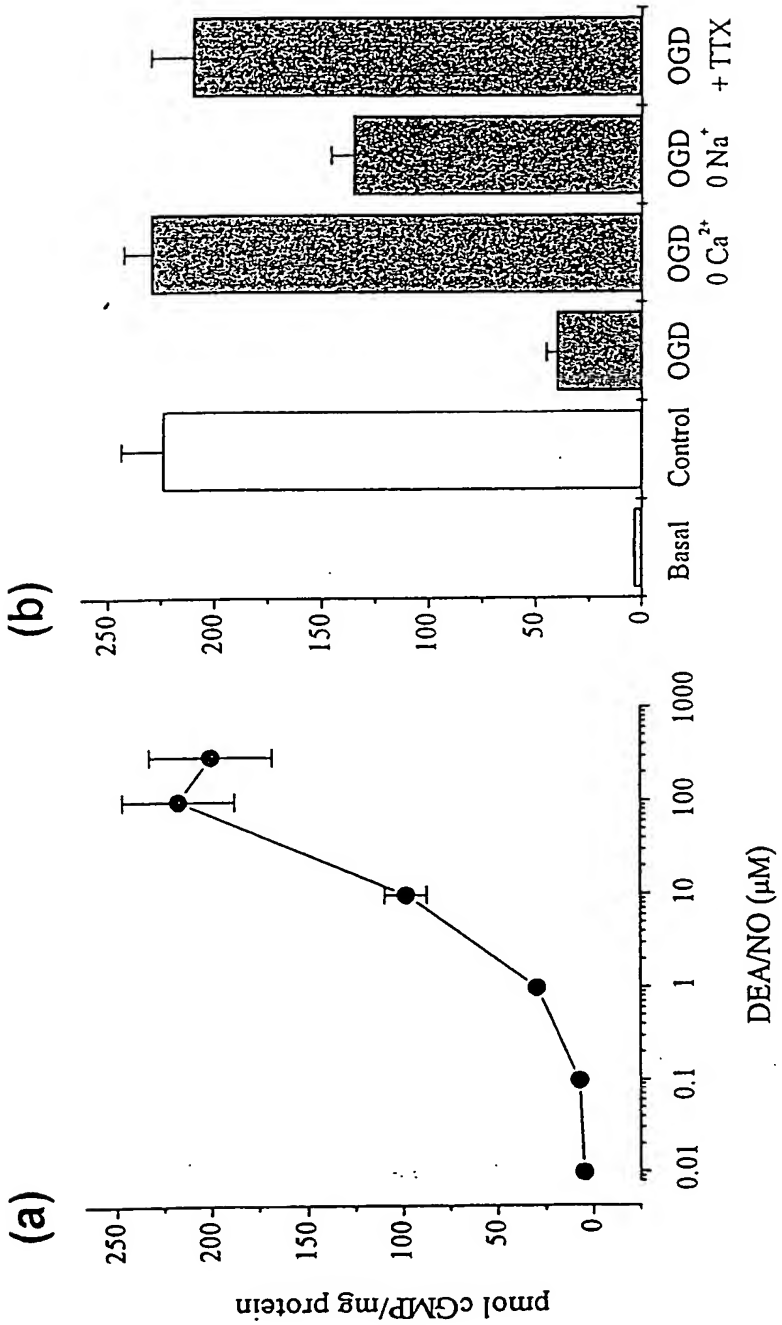
(a)
pmol cGMP/mg protein
250
200
150
100
50
0
0.01
0.1
1
10
100
1000
DEA/NO (μM)
(b)
250
200
150
100
50
0
Basal
Control
OGD
OGD
0 Ca2+
OGD
0 Na+
OGD
+ TTX

Figure 1

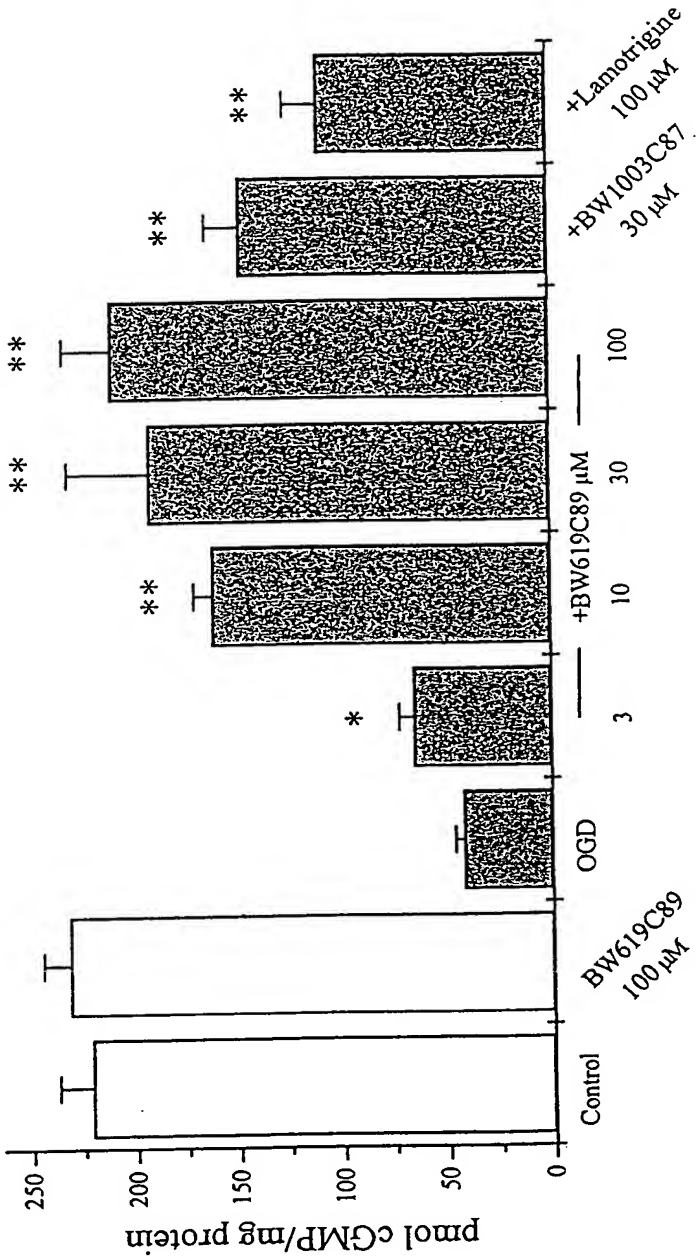
pmol cGMP/mg protein
250
200
150
100
50
0
Control
BW619C89 100 µM
OGD
+BW619C89 µM
3
10
30
100
+BW1003C87 30 µM
+Lamotrigine 100 µM
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Figure 2

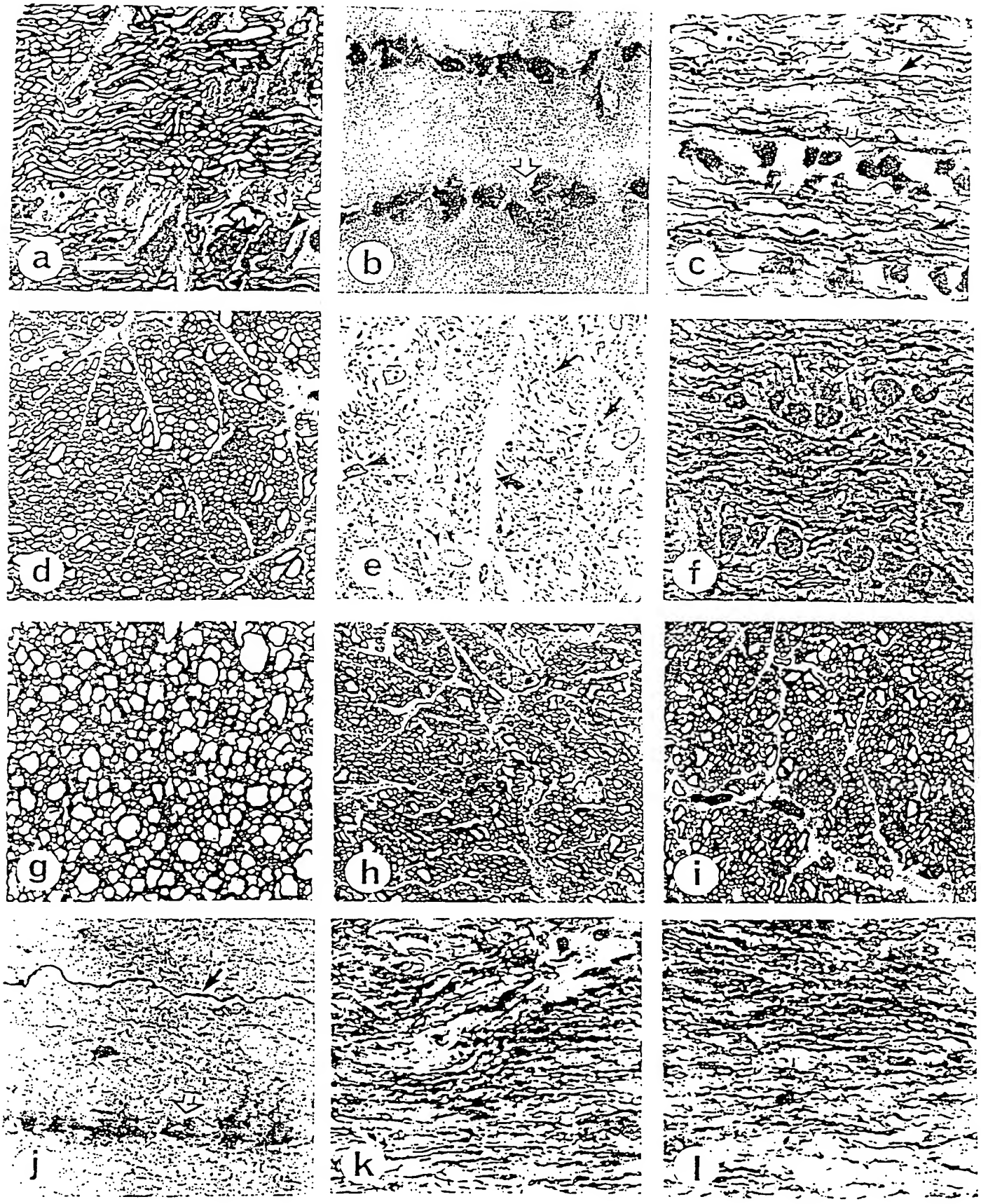

Figure 3